RNA/DNA RATIOS IN THE ESTIMATION OF GROWTH STAGES OF OCEANIC ZOOPLANKTON POPULATIONS

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THESIS

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by

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This study indicates that RNA/DNA ratios are related to growth stages of the splash zone copepod Tigriopus californicus. These ratios have the potential to be applied to models which relate zooplankton populations to the food chain and therefore to the sound scattering parameters which are of great interest to the Navy.

RNA/DNA Ratios in the Estimation of Growth Stages of Oceanic Zooplankton Populations

by

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requirements for the degree of

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RNA concentrations are found by measuring the ultraviolet (UV) absorption of its purine and pyrimidine base groups. Interference from protein in the RNA measurement is accounted for by employing differential UV absorption. DNA concentrations are found by measuring the UV absorption of an indole-deoxyribose adduct.

This study indicates that RNA/DNA ratios are related to growth stages of the splash zone copepod Tigriopus californicus. These ratios have the potential to be applied to models which relate zooplankton populations to the food chain and therefore to the sound scattering parameters which are of great interest to the Navy.

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I. INTRODUCTION

From the Navy's point of view sound transmission is a most important physical characteristic of the sea. Detectional, navigational, and weapons sensors all rely upon this phenomenon. Studies of sound in the sea reveal the presence of scatterers that can reduce the effectiveness of acoustic sensors [Tucker, 1957]. This sound scattering appears as a volume reverberation term and reduces the recognition differential level associated with a specific sensor and a specific target. This scattering has been associated with zooplankton in the sea [Traganza and Stewart, 1973].

Zooplankton levels are not constant in that there are marked diurnal, seasonal, and regional variations [Cushing, 1959]. While zooplankton biomass variability does not cause fluctuations in low frequency volume reverberation levels directly, it indirectly influences the levels through the food chain. Therefore, a measurement of the rate of biomass change could lead to a prediction of biological volume reverberation fluctuations.

Models designed to estimate the growth rate of zooplankton populations are based on field measurements of a change in biomass. This type of measurement requires two biomass samples over a period of time. Current techniques are plagued with too many variables to make these measurements

anything but a rough estimate [Pease, 1973]. In addition to the variability, the amount of time, equipment, and personnel needed to produce these measurements make this method a very limited procedure; that is, not an *in situ* measurement.

In the search for better methods of biomass measurement the field of marine biochemistry holds a great potential [Riley and Chester, 1971]. Chemical measurements may be made quickly, accurately, and with certainty. Early research in this concern approaches the idea of relating a cellular constituent like ribonucleic acid or deoxyribonucleic acid to a population growth rate [Sutcliffe, 1965]. A specific chemical parameter has not yet been accurately related to predicting growth rates in marine zooplankton. It is to this end that this research was undertaken.

II. HISTORICAL

Current thought holds that RNA is a necessary precursor to protein synthesis [Brachet, 1960; Roth, 1961; Sutcliffe, 1965; Miller, 1969; Hoagland, 1959; Clark and Marcker, 1968; Yanofsky, 1967; Gale, 1956; Kornberg, 1960]. To better understand this relationship between RNA and protein synthesis, it is necessary to examine the basic chemistry of RNA, DNA, and protein molecules.

Early cytochemical observations showed that RNA was abundant in rapidly growing cells such as onion root tips. At the same time it was found that cells with a high physiological activity but with a slow growth rate such as heart muscle, contained little RNA. THese observations lead to the conclusion that cells that synthesize large amounts of protein contain large amounts of RNA. Qualitative observations have shown that there is a direct correlation between RNA synthesis and the synthesis of proteins in populations of exponentially growing cells [Brachet, 1959].

The role of RNA is inseparably related to the equally important DNA. The Nobel prize winning work of Watson and Crick in discovering the structure of the DNA molecule was the basis for understanding the DNA control and continuance of cell functions [Crick, 1968]. DNA is the carrier of the genetic message from one generation to the next, and thus is the basis for the control and continuance of cellular

functions. This means that it is DNA that ensures that progeny carry on similar functions over many generations [Kornberg, 1960]. These two DNA functions of control and continuance are executed by the actions of RNA but can be best understood by examining the basic structure of a DNA molecule.

DNA and RNA molecules are composed of an ordered sequence of basic units known as nucleotides. A nucleotide is structured from a sugar, a nitrogenous base, and a phosphate group as illustrated in Figure 1. Two linear chains of complimentary nucleotides bonded together in the form of a double helix are the basis for DNA molecular structure. The two complimentary chains of nucleotides are joined across the central axis of the double helix by hydrogen bonding between base pairs (see Figure 2). RNA structure is similar except for the following: ribose replaces deoxyribose, uracil replaces thymine, and RNA occurs as a single, coiled chain.

DNA's abilities to continue and direct cell functions originate with the nature of the bonding between bases in the complimentary nucleotides in the DNA helix. The physical size of the different bases dictates that purines can only bond to pyrimidines and still fit the DNA helical structure (see Figure 3).

Linear groups of 3 nucleotides (a codon) occuring along one strand of a DNA molecule can be associated to a specific

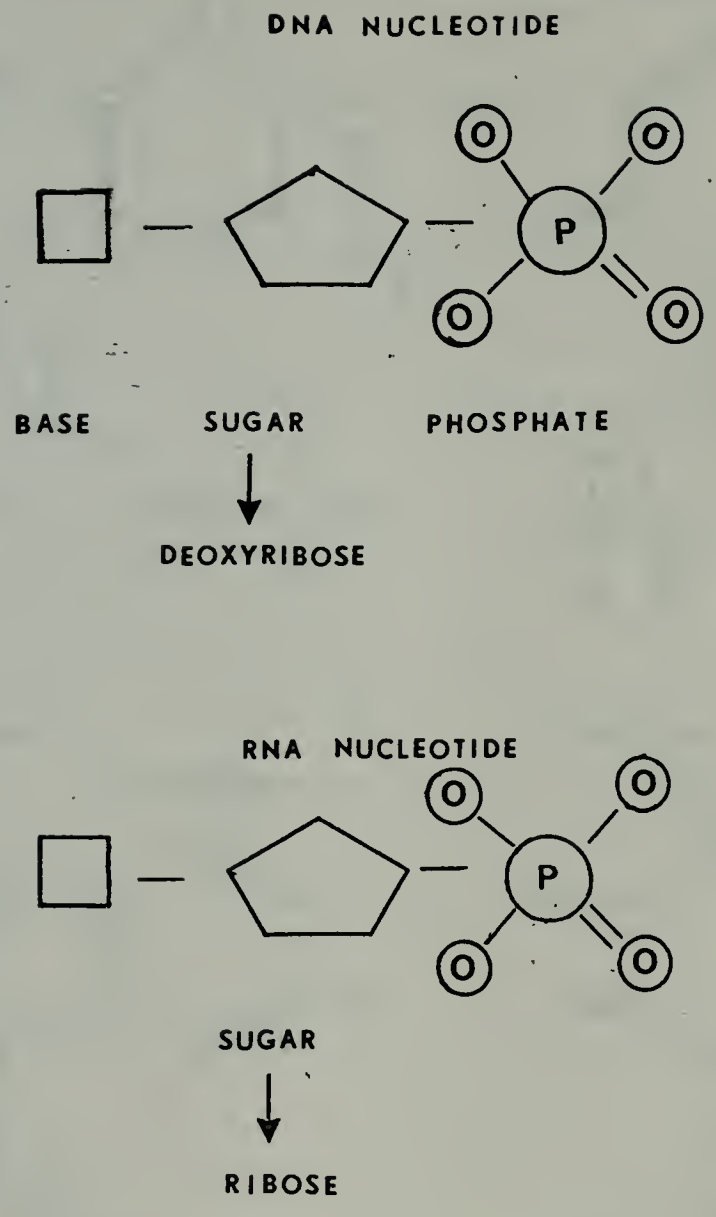


Figure 1. The basic three-part structure of a DNA and an RNA nucleotide.

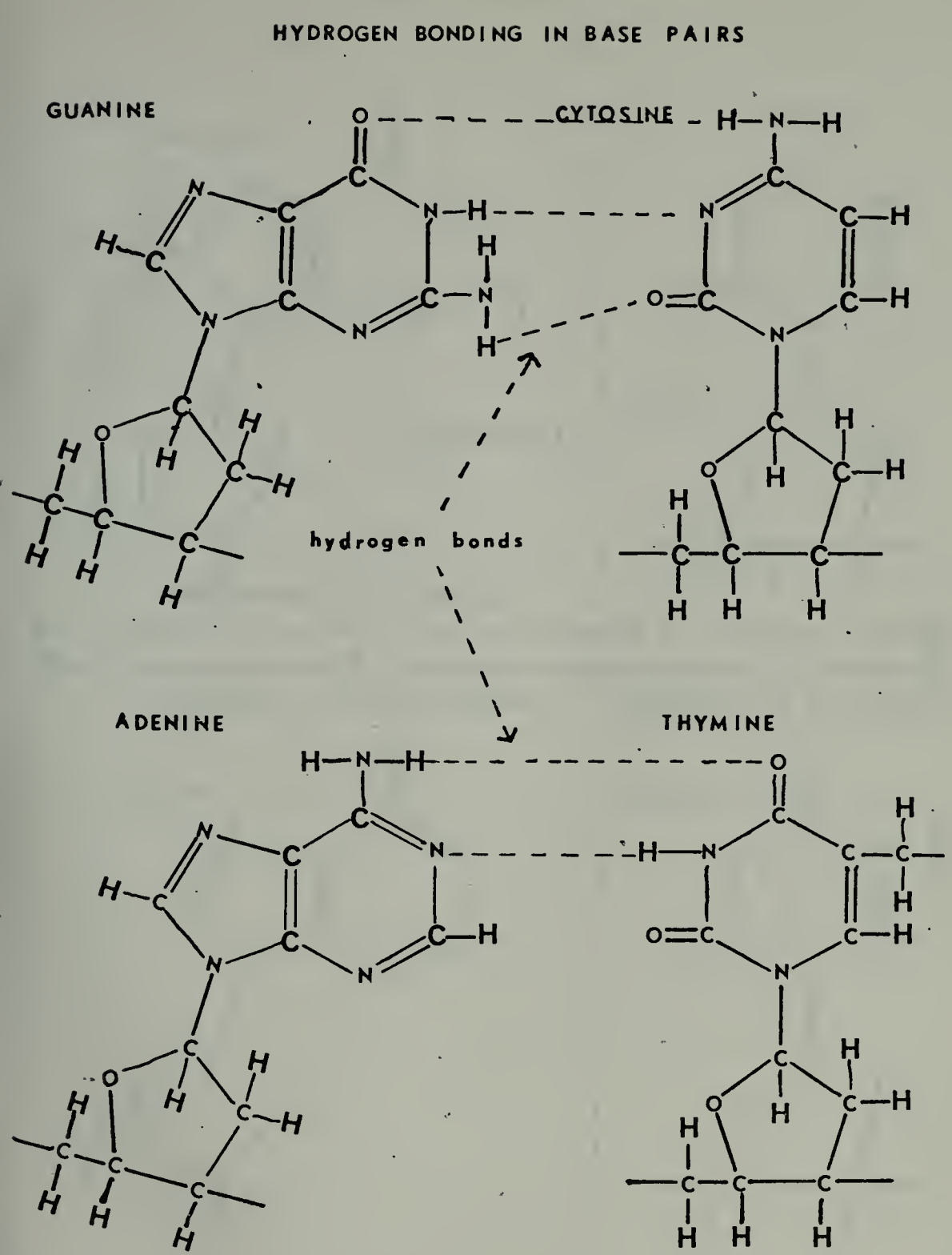


Figure 2. Hydrogen bonding as it occurs between complimentary base pairs in the DNA double helix or between the different forms of DNA and RNA as found in protein synthesis.

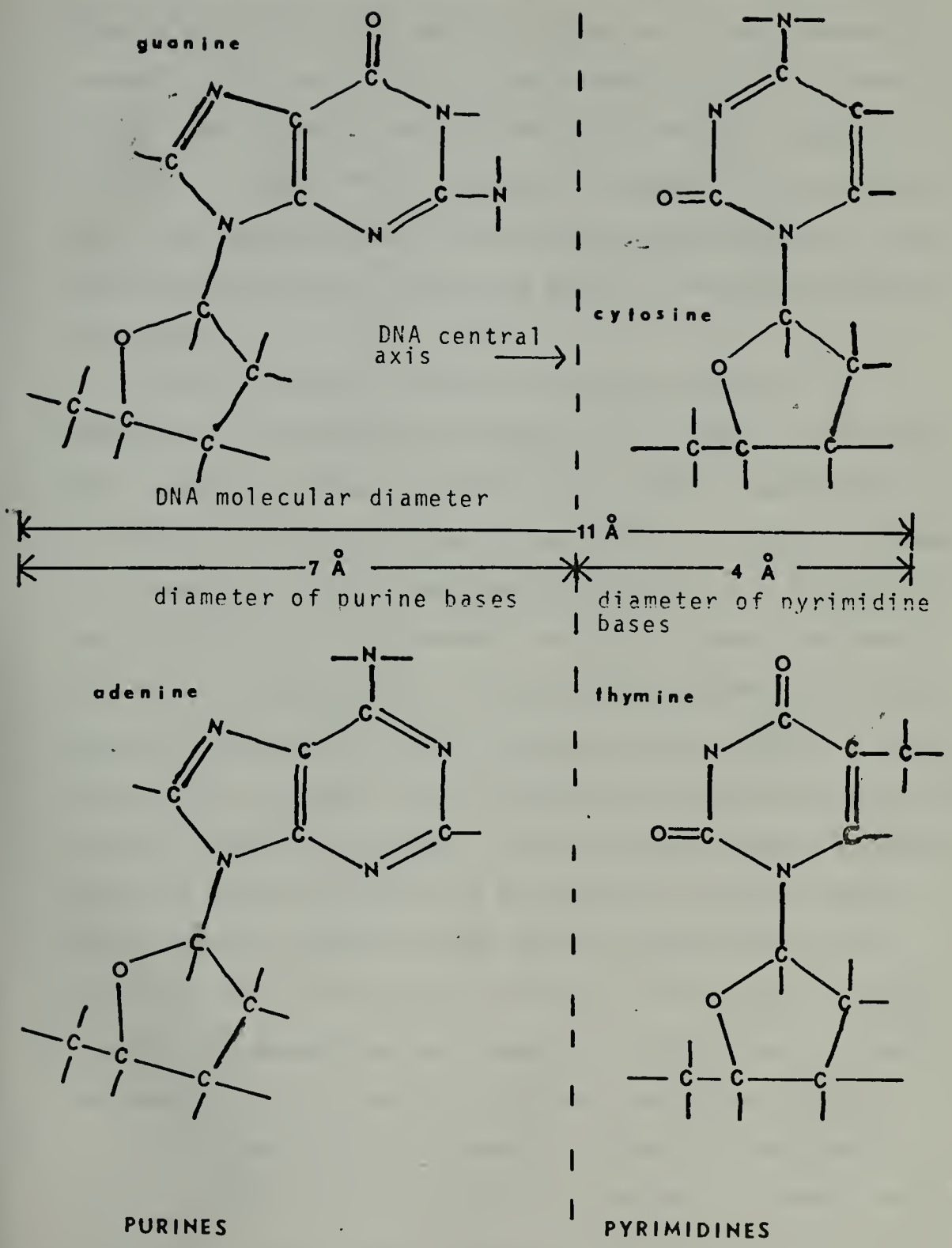


Figure 3. The physical size relationship between complimentary base pairs and the DNA molecule is such so that only purines can bond to pyrimidines and vice-versa.

amino acid from which proteins are built (see Figure 4). Thus a section of DNA molecule with the proper number of codons can be associated to any protein molecule [Kornberg, 1960]. DNA directs the building of a protein molecule indirectly through mRNA, tRNA, and ribosomal RNA (see Figure 5). The complimentary bonding between base pairs in DNA and RNA molecules is the basis for this method of protein structure.

In any given cell the more proteins that are to be synthesized, the more RNA that must be present to perform this function. Thus a measure of the RNA concentration in a cell will give some idea of the rate of protein synthesis.

Essentially DNA is entirely located in the nucleus of the cell (except in bacterial and uiral forms) and forms the genes characteristic of that cell species. Any given mature organism will have a characteristic number of genes (diploid), the same for all mature cells excepting sex cells in that organism (haploid). With the same number of genes, the same amount of DNA will be found in the cells and a characteristic amount of DNA can be associated to any organism at a given time in its life. This idea provides a basis for measuring the number of cells present in an organism or in a population [Mirsky and Osawa, 1961].

Many populations go through a sigmoid growth curve [Miller, 1969], with four distinct stages of growth (see Figure 6). Each stage is characterized by its particular

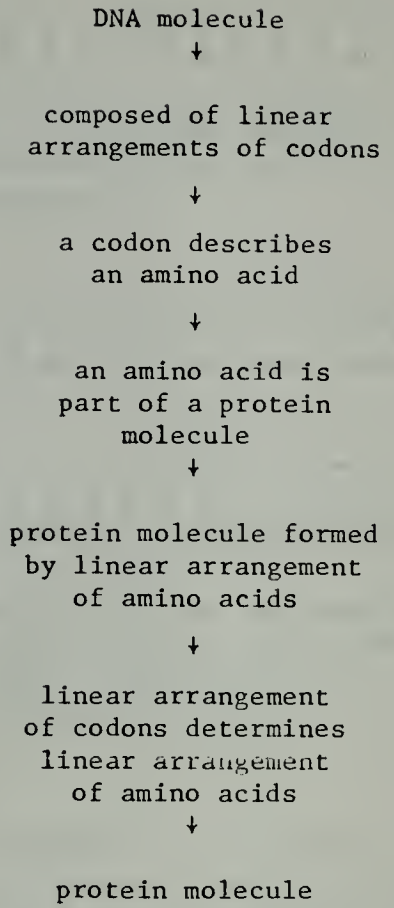


Figure 4. The logical sequence of the formation of a specific amino acid from a specific order of codons on a DNA strand.

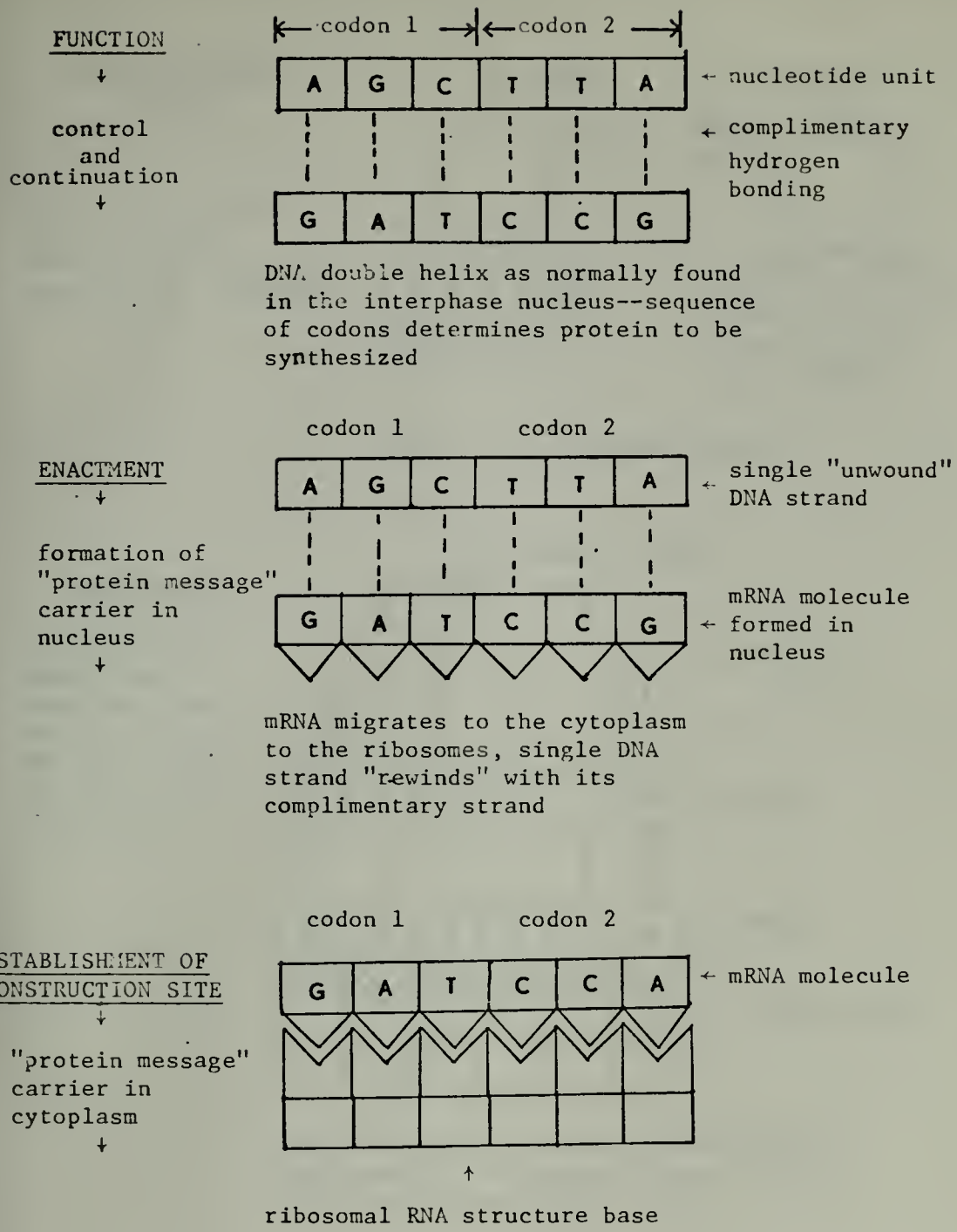


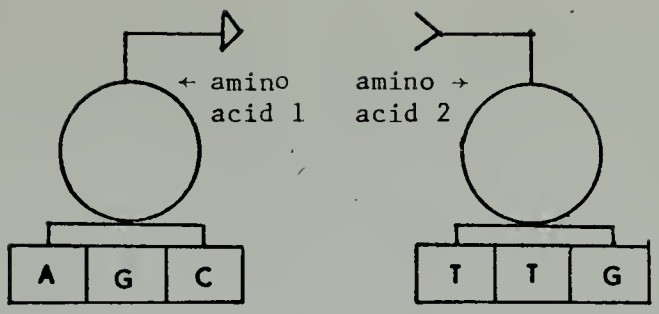
Figure 5(a). The beginnings of protein synthesis from the "protein message" carried on the DNA molecule to the formation of a mRNA molecule that corresponds to the same protein.

RAW MATERIALS SUPPLY

↓

attachment of amino acids to tRNA molecules and migration to ribosomes

↓



tRNA molecule anti-codon 1

tRNA molecule anti-codon 2

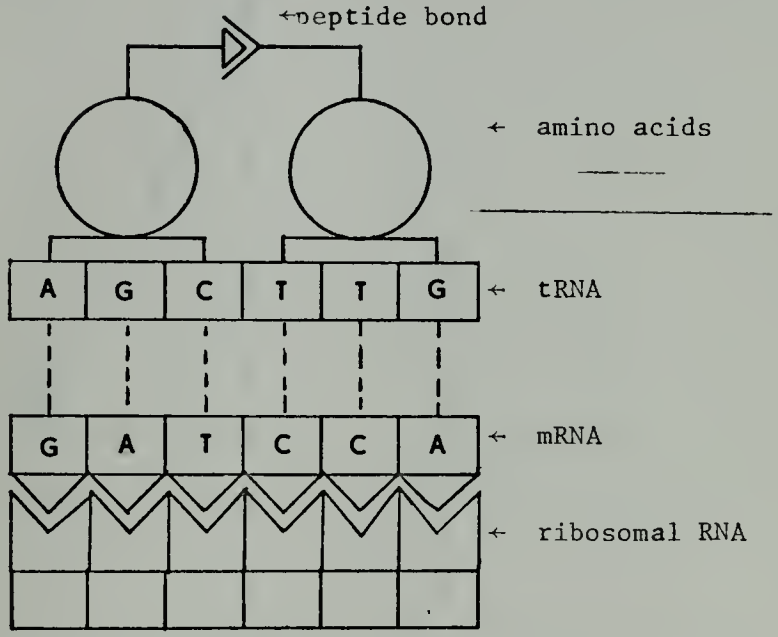
This is a cytochemical process

CONSTRUCTION

↓

formation of protein molecule resulting from pairing of codons and anti-codons on the ribosomal RNA structure

↓



proper allignment of amino acids results from specific allignment of tRNA molecules along the mRNA molecule's axis

Figure 5(b). Protein synthesis from the placement of the mRNA molecule on the ribosome to the formation of tRNA molecules that attach to their specific amino acids and then line up along the mRNA molecule causing the proper sequence and positioning of amino acids to occur resulting in the formation of a protein molecule.

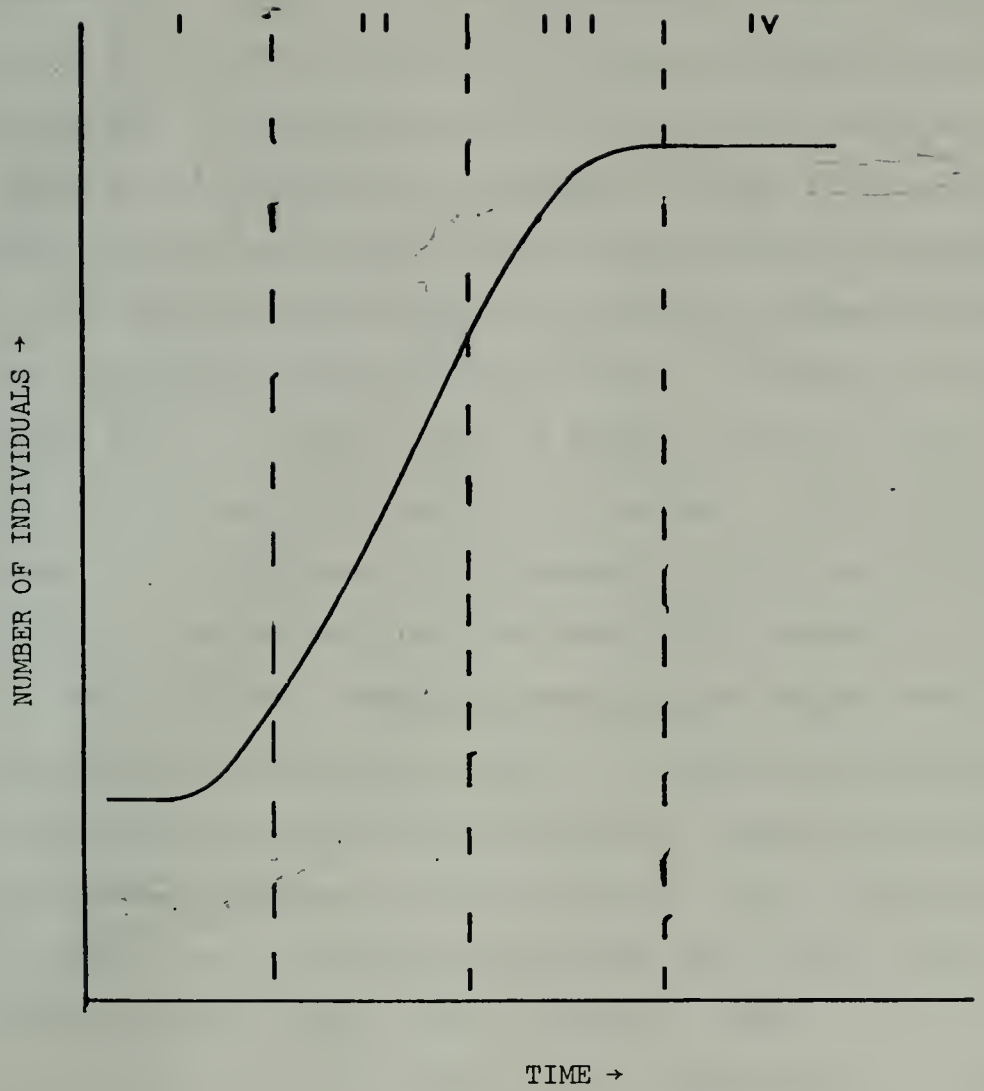


Figure 6. Number of individuals versus time for an idealized population growth exhibiting Sigmoid growth characteristics.

type of growth. Stage I is an initial growth stage with a small number of individuals, but with a large progeny potential. Stage II is the exponential growth stage where young are rapidly produced and the old members die off. Stage III is a maturation period where the young age and where no net progeny are produced. Stage IV growth depends upon the population dynamics of the particular species. If the number of new progeny equals the number of deaths, the population reaches steady state. Likewise, if the death rate or removal rate is higher than the birth rate, the population will decrease in number. It is during this stage that environmental interactions with the population have a large effect upon the population numbers.

An individual organism goes through stages very similar to sigmoid population dynamics. A newly born individual goes through an initial hyperplastic stage characterized by a rapid increase in cell numbers. This is followed by a mixed stage in which hyperplastic and hypertrophic (cytoplasmic) growth occurs [Miller, 1969].

It has been shown that protein synthesis is a necessary precursor to cell growth, and that RNA production is directly related to the rate of protein synthesis. Thus one could expect to find the highest relative concentration of RNA in the fastest growing individuals, Sigmoid stage II or mixed hyperplastic and hypertrophic stages; with decreasing relative concentrations of RNA being found in older and

slower growing individuals. Furthermore, so basic a metabolic process, such as protein synthesis, is not likely to be species specific within broad limits. Thus the RNA-growth relationship from individual organisms should be applicable to populations of organisms, and RNA concentrations as normalized with DNA concentrations might permit a prediction of growth rate and growth stage, even when applied to mixed populations, such as zooplankton.

In 1965 W. H. Sutcliffe, Jr. was one of the first researchers to obtain significant results indicating RNA-growth relationships [Sutcliffe, 1965]. Sutcliffe used synchronous cultures of Artemia salina and Nassarsisus obsoletus as test organisms. He made RNA-to-dry-weight determinations on the amphipod Orchestia platensis and used this result to produce a growth rate-RNA relationship. He then applied his growth rate model to the mud snail and brine shrimp cultures' actual growth and found that a reasonable correlation existed between these cultures and cultures of O. platensis. Sutcliffe then concluded that the RNA-growth rate relationship could be useful in predicting growth rates in other psecies or mixed populations. Sutcliffe extended his early work by comparing a collection of curves of growth rate-RNA relationships that involved 24 species (see Figure 7). Again he suggested that this data showed a positive relationship between growth rate and RNA concentration [Sutcliffe, 1969].

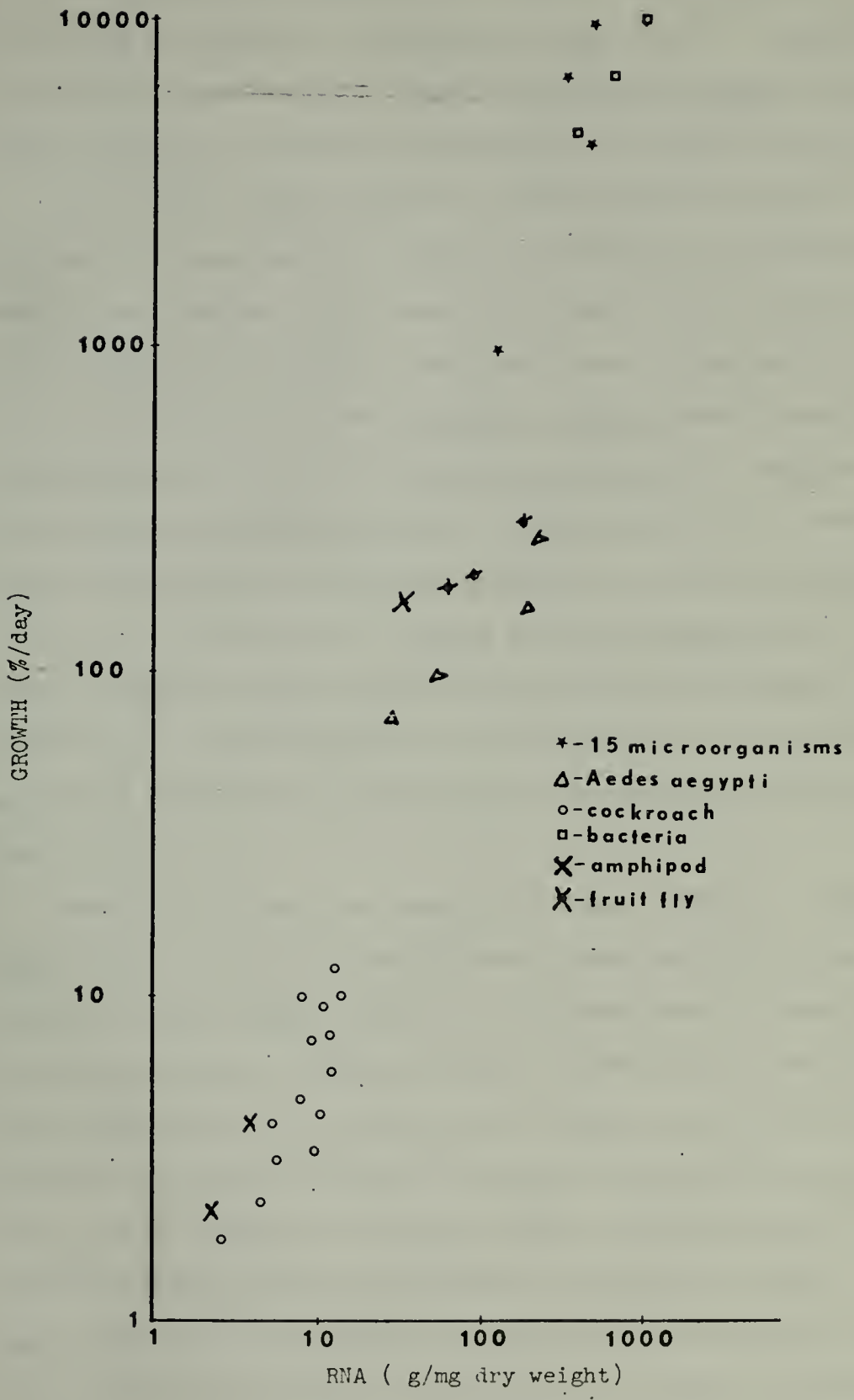


Figure 7. Growth (% per day) versus RNA (g/mg dry weight) for six species (after Daag and Littlepage, 1972).

In contrast to Sutcliffe's work are the results of the work done by Dagg and Littlepage [Dagg, 1972]. Dagg and Littlepage collected the copepod Euchaeta elongata and used Artemia salina to develop synchronous cultures from which to work. They made analysis of RNA versus dry weight, protein, DNA,and percent growth to examine the RNA-growth rate relationship. The results of their work showed that neither Sutcliffe's 1965 RNA-growth rate equation nor their own equation derived from Artemia salina growth, accurately predicted growth rates for Euchaeta elongata. Dagg and Littlepage concluded that even though there was a statistically significant relationship between growth rates and RNA/dry wt. ratios in A. salina and E. elongata, the wide range of growth rates associated with a small range of RNA/dry wt. ratios caused the relationship to lack sufficient acuity to be used in a RNA-growth rate growth prediction (see Figure 8).

Sutcliffe's work was tested by Pease [1968]. Pease found that Sutcliffe's relationship was valid only during the most rapid growth stage of the experimental organism. He concluded that the growth rate to RNA relationships were specific only to organisms in their most rapid growth stages, and that the rest of the time they were related only to the organism from which they were derived. Notwithstanding their difference of opinion on the applicability of the general trend in the RNA-growth rate relationships as seen in the results of Pease, Sutcliffe and

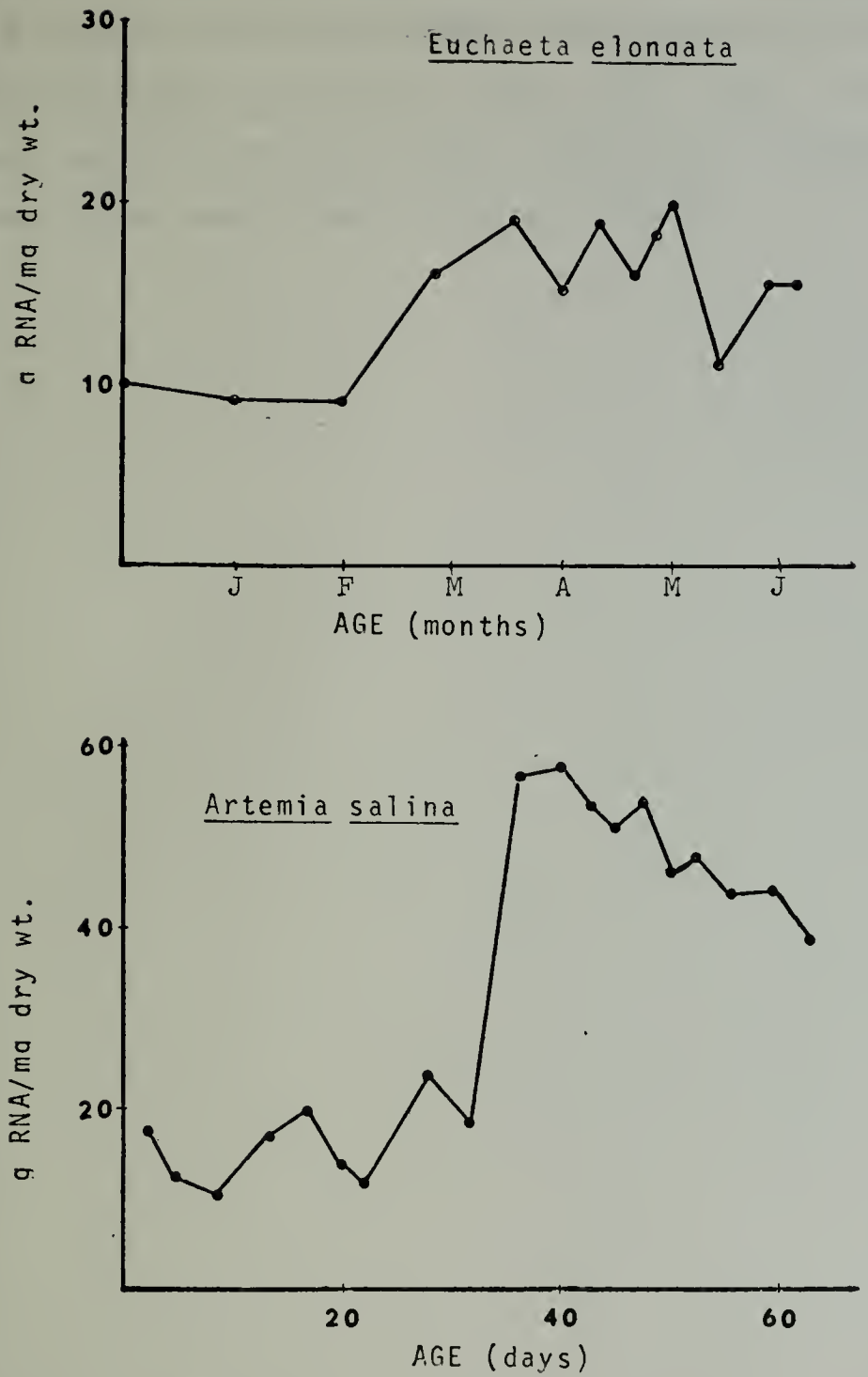


Figure 8. Micrograms RNA per mg dry weights versus age (days) for cultures of Artemia salina and Euchaeta elongata (after Dagg and Littlepage, 1972).

Dagg and Littlepage, is the work of a host of others, using a spectrum of organisms [Leick, 1968; Vickers and Mitlin, 1965-66; Haines, 1973; Lay, 1965; Bulow, 1970]. The direct application of RNA growth rate predictions to marine zooplankton populations is as yet untested.

III. EXPERIMENTAL PROCEDURE

A. COLLECTION AND HANDLING OF T. CALIFORNICUS

Populations of Tigriopus californicus were collected in splash pools found above the mean high-water mark. The pools were found along the rocks that line the beach around Lover's Point at the southern end of Monterey Bay, California. The animals were scooped from the pools with a number 10 plankton bucket. Presumably natural food found in the splash pools was transported with the copepods. Identification of the species was simplified insofar as T. californicus is the only species of marine copepod found in splash pools above the mean high-water mark along Monterey Bay and that they possess a distinctive reddish-orange color [Egloff, 1966]. Microscopic examination confirmed the visual analysis and alos indicated that the catches were homospecific.

Once collected, the copepods were kept in plastic containers at room temperature (ca. 23°C) near a source of sunlight (the copepods are algae feeders). Those copepods that were to be kept over a period of time longer than a week were refrigerated at 8°C.

A Pasteur pipette with a length of thin tubing for applying suction by mouth was used to separate experimental groups, e.g., gravid females, from the others. Immediately prior to analysis the copepods were filtered on to a millipore 0.45 μm HA filter and washed with distilled water.

The organisms were "dried" for weighing by being scraped onto a dry Whatman number 3 filter paper. They were then manually separated from the debris into a plastic weighing dish and weighed to the nearest tenth of a milligram (mg).

B. PRE-ANALYSIS

A glass tissue grinder, distilled water, 0.6 normal (N) perchloric acid (PCA), and 1.2 N PCA were placed over ice prior to analysis. Two water baths were set up sufficiently far in advance so that they had stabilized at temperatures of 37°C and 100"C at the time of analysis.

A 200 mg "semi-dry weight" sample of copepods, ca. 1000, was taken from the experimental group being analyzed and was placed into an ice packed tissue grinder with ice-cold distilled water. The copepods were ground for 10 minutes or until a fine homogenate was formed.

C. SAMPLE ANALYSIS

The Schmidt-Thannhauser method [Schmidt, 1945] as modified by Munro and Fleck [Munro, 1969] was used to separate and measure the RNA fraction of the sample. Interfering protein was checked by using differential ultraviolet (UV) spectrometic techniques [Munro, 1969]. DNA concentration was found by employing Ceriotti's indole method [Cerriotti, 1952] as modified by Munro and Fleck [Munro, 1969].

D. PREPARATION OF STANDARDS

DNA stock solution standards were prepared by dissolving 20 mg of calf thymus DNA in 50 milliliters (ml) distilled water. Five ml of 1 N NaOH were added to the DNA to aid the dissolution of the thymus strands by ensuring the separation of DNA molecules in a basic solution. It took 30 minutes for the thymus to dissolve, as heating is prohibited to prevent the breakdown of the DNA molecules. The stock solution was kept refrigerated until needed. Working solutions were prepared by diluting the stock standard 1:25 with distilled water to produce a standard solution of 32 micrograms (µg) DNA/ml from which multiple dilutions were made.

A RNA stock solution standard was prepared by dissolving 32 mg of yeast RNA in 1 liter (l) of 0.1 N PCA. The solution was heated in a boiling water bath to dissolve the RNA. The stock solution containing 32 µg of RNA/ml was refrigerated until needed. Working solutions were made by diluting 5 ml portions of the stock solution with 0.1 N PCA.

A protein standard was prepared by dissolving 5 mg bovine serum albumin in 50 ml distilled water. This produced a stock solution of 100 µg protein per ml of solution. Repeated analysis of all of the stock standards over a period of three months indicated that the standards did not deteriorate to any detectable extent.

E. PRECIPITATION OF RNA AND DNA

Two and one-half ml of ice-cold 0.6 N PCA were added to 50 ml of copepod homogenate in a centrifuge tube. The centrifuge tube contents were mixed and allowed to stand for ten minutes. The mixture was centrifuged and the supernatant was discarded. The residue was washed with 5.0 ml 0.2 N PCA. The supernatant was again discarded after centrifuging. The residue wash was repeated with another 5.0 ml 0.2 N PCA to ensure precipitation of all of the nucleic acid molecules. The supernatant was drawn off with a Pasteur pipette and discarded (see Figure 9).

F. HYDROLYSIS OF RNA MACRO-MOLECULAR STRUCTURE

Four milliliters 0.3 N KOH was added to the residue and this combination was heated for one hour at 37°C. Munro and Fleck, 1969, showed that the least amount of protein carry-over into the RNA solution will occur at this combination of temperature and time (there is some danger of digesting protein molecules at higher temperatures). Two and one-half milliliters of cold 1.2 N PCA were added and the mixture was stirred. The mixture was cooled for ten minutes in an ice bath and was then centrifuged. The supernatant was held for RNA determination and the residue was used for DNA determination (see Figure 10).

G. DIGESTION AND MEASUREMENT OF DNA

The precipitate from above was washed twice with 5 ml of 0.2 N PAC and the washings combined with the supernatant

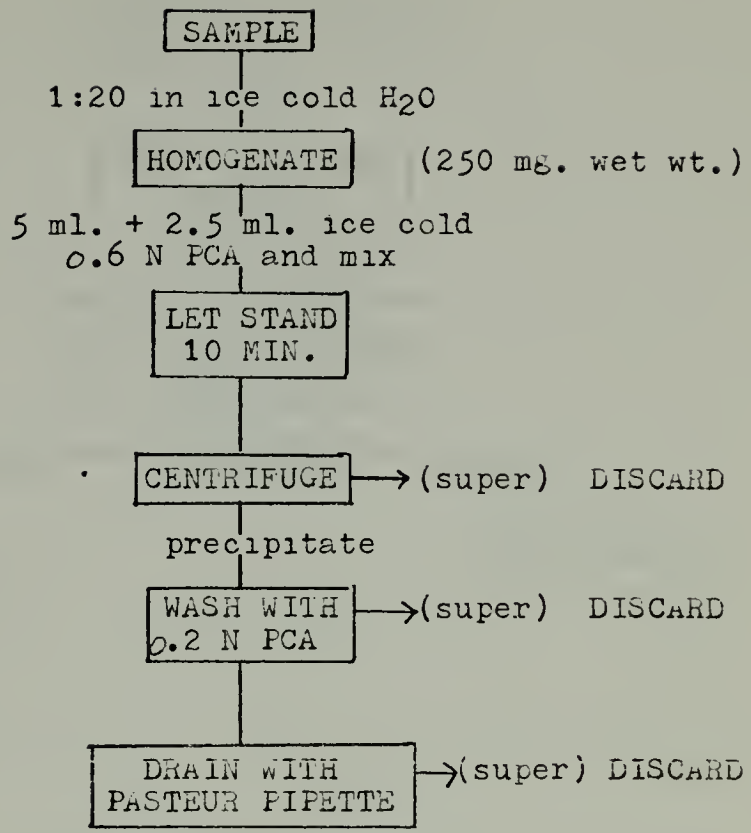


Figure 9. Perchloric acid (PCA) precipitation of nucleic acid, phospholipids, and tissue protein from a homogenate of T. californicus.

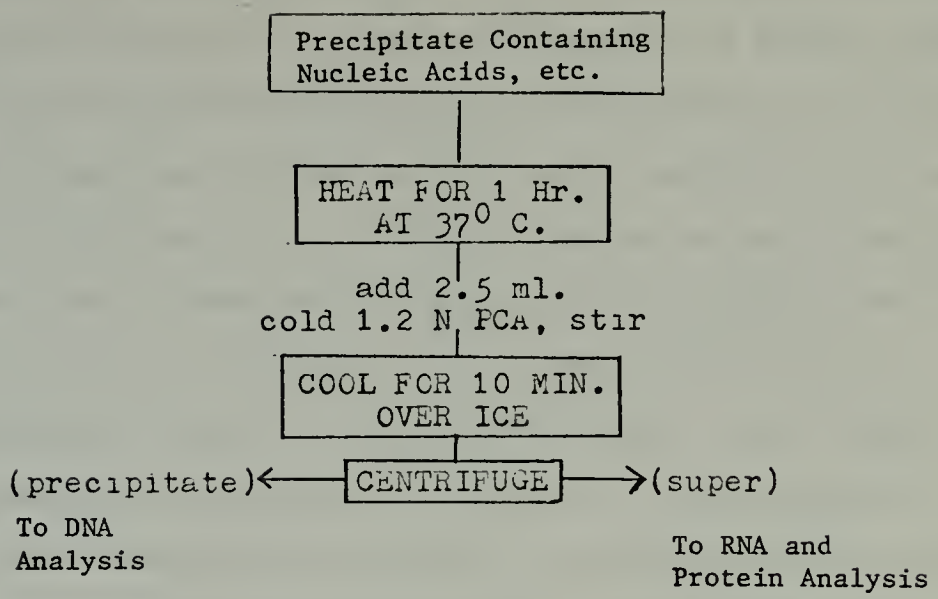


Figure 10. The hydrolysis of RNA from the PCA precipitate of the T. californicus homogenate by controlled digestion and the separation of RNA from DNA by centrifugation and extraction.

from above and saved for the RNA analysis. Four milliliters of 0.3 N KOH was added to the precipitate and the mixture was heated to a high temperature in a Bunsen flame until the precipitate was digested indicating that the DNA molecules had been broken up into basic nucleotide units. Twelve milliliters of 0.3 N KOH was added to the digestion and the total volume was brought up to 50 ml with distilled water. To 2.0 ml of this solution was added 1.0 ml of concentrated HCl and 1.0 ml of 0.04% indole color reagent. The mixture was heated for 15 minutes in a boiling water bath then cooled in running water and extracted three times with $CHCl_3$. The last extraction was centrifuged at 300 revolutions per minute for five minutes before the $CHCl_3$ was removed. The extract was discarded and the absorbance of the aqueous layer was read at 490 nm to find the relative DNA concentration (see Figure 11).

H. MEASUREMENT OF RNA AND THE PROTEIN CHECK

Ten milliliters 0.6 N PCA was added to the RNA supernatant which was combined with the RNA washings from the DNA digestion step. The solution was made to 100 ml with distilled water. The absorbance of the solution was read at 260 nm in order to measure the relative RNA concentrations.

The optical absorbance was also read at 280 nm to obtain a differential protein value. Munro and Fleck, 1969, showed that RNA has a major absorption peak at 260 nm, while pure bovine serum protein has its major absorption peak at 280 nm.

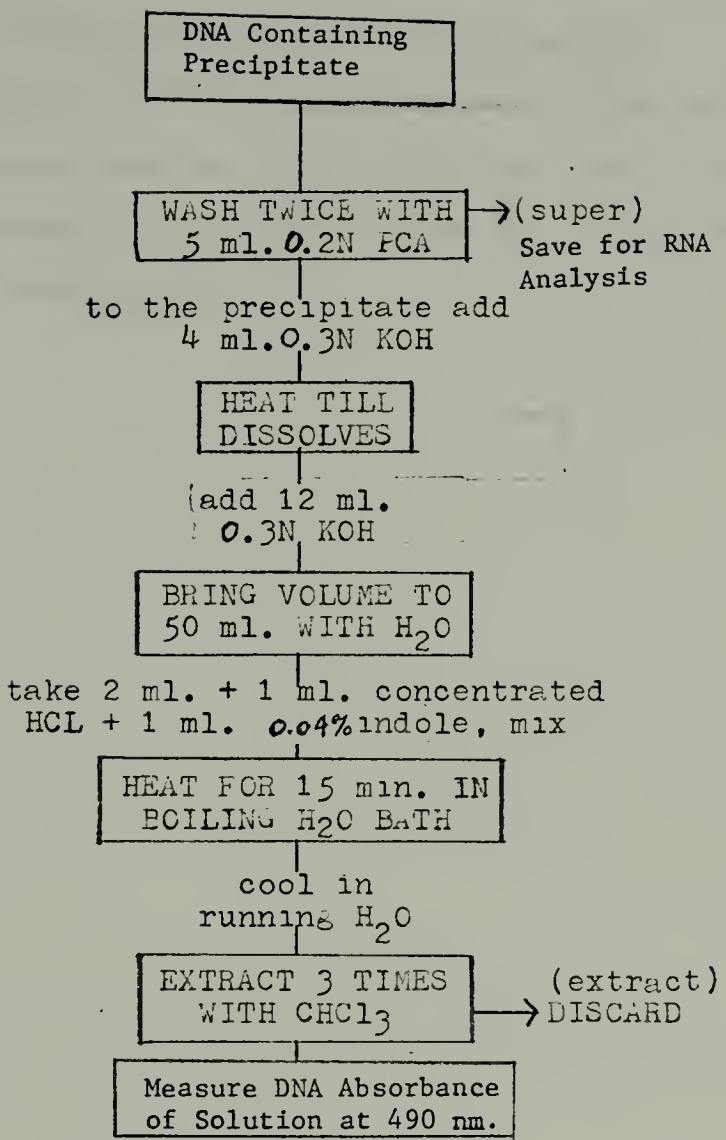


Figure 11. The digestion of DNA from the PCA precipitate after RNA was extracted off, and the spectrophotometric measurement of the deoxyribose-indole adduct formed in the process.

They also ran a series of absorption spectra for different RNA, RNA and protein, and protein samples, all of known concentrations. From these values they determined readings at 280 nm that would indicate significant amounts of protein carried over into the RNA extract that would give erroneous relative RNA concentrations. Therefore the reading is made at 280 nm to ensure that a protein free RNA extraction had been performed (see Figure 12).

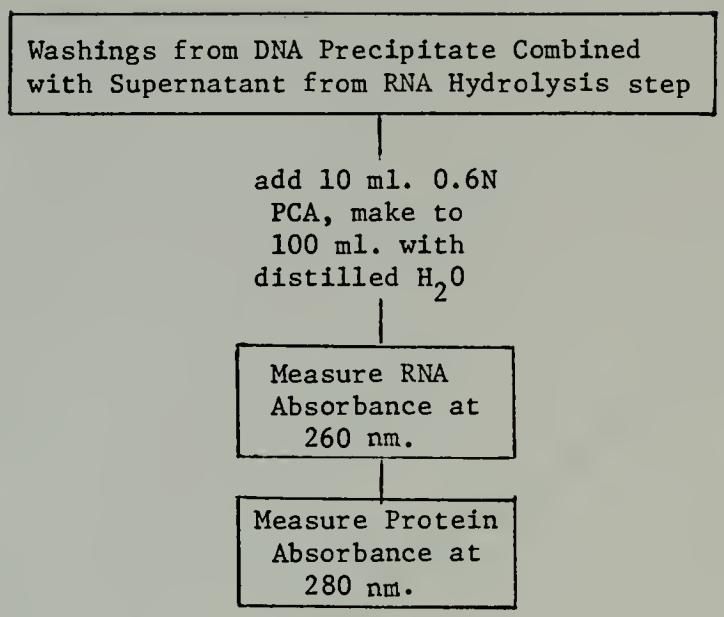


Figure 12. The UV absorption of the RNA bases and the differential UV absorption of carried-over proteins from the combined supernatant and washings of the PCA precipitation of DNA and RNA hydrolysis step.

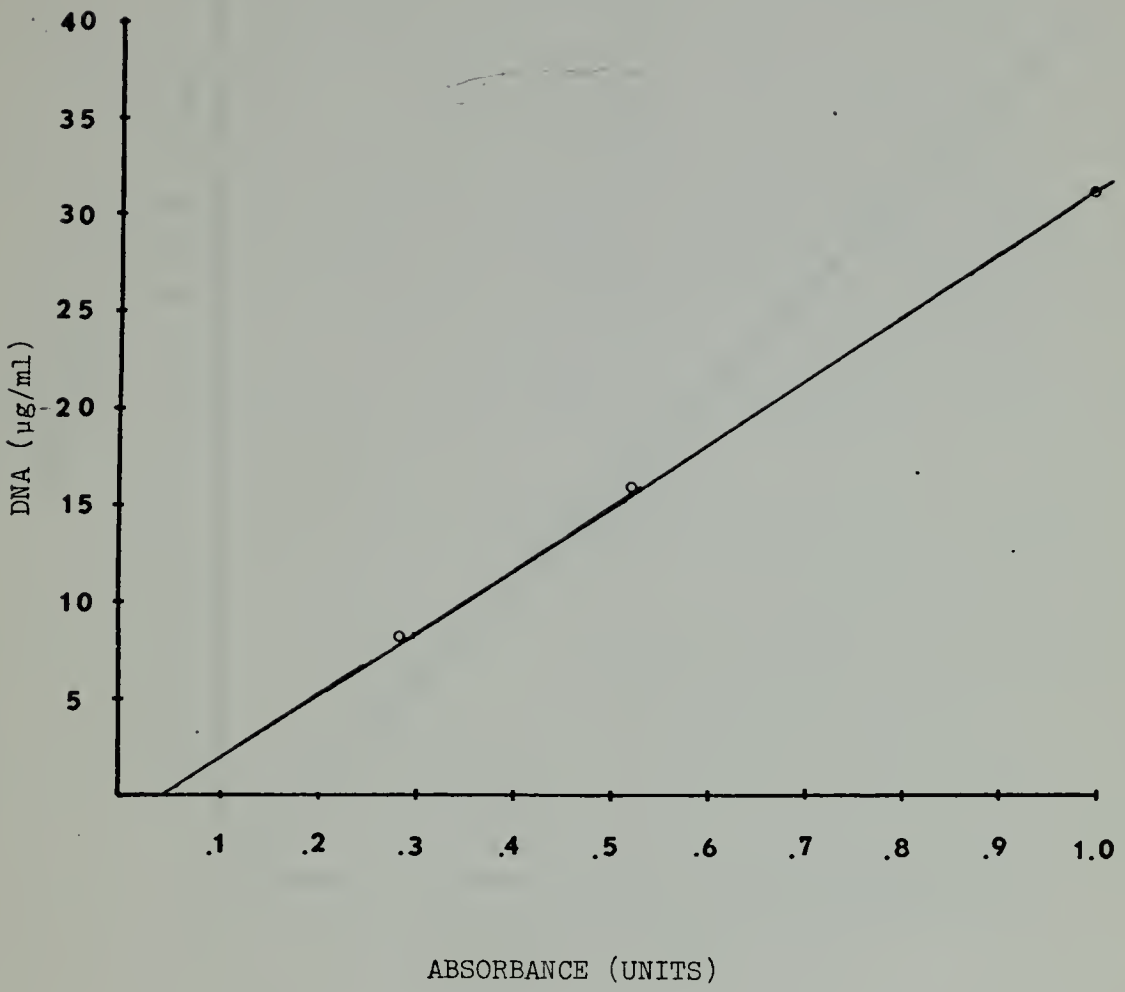


Figure 13. DNA (µg/ml versus absorbance (units) for a calf thymus DNA standard.

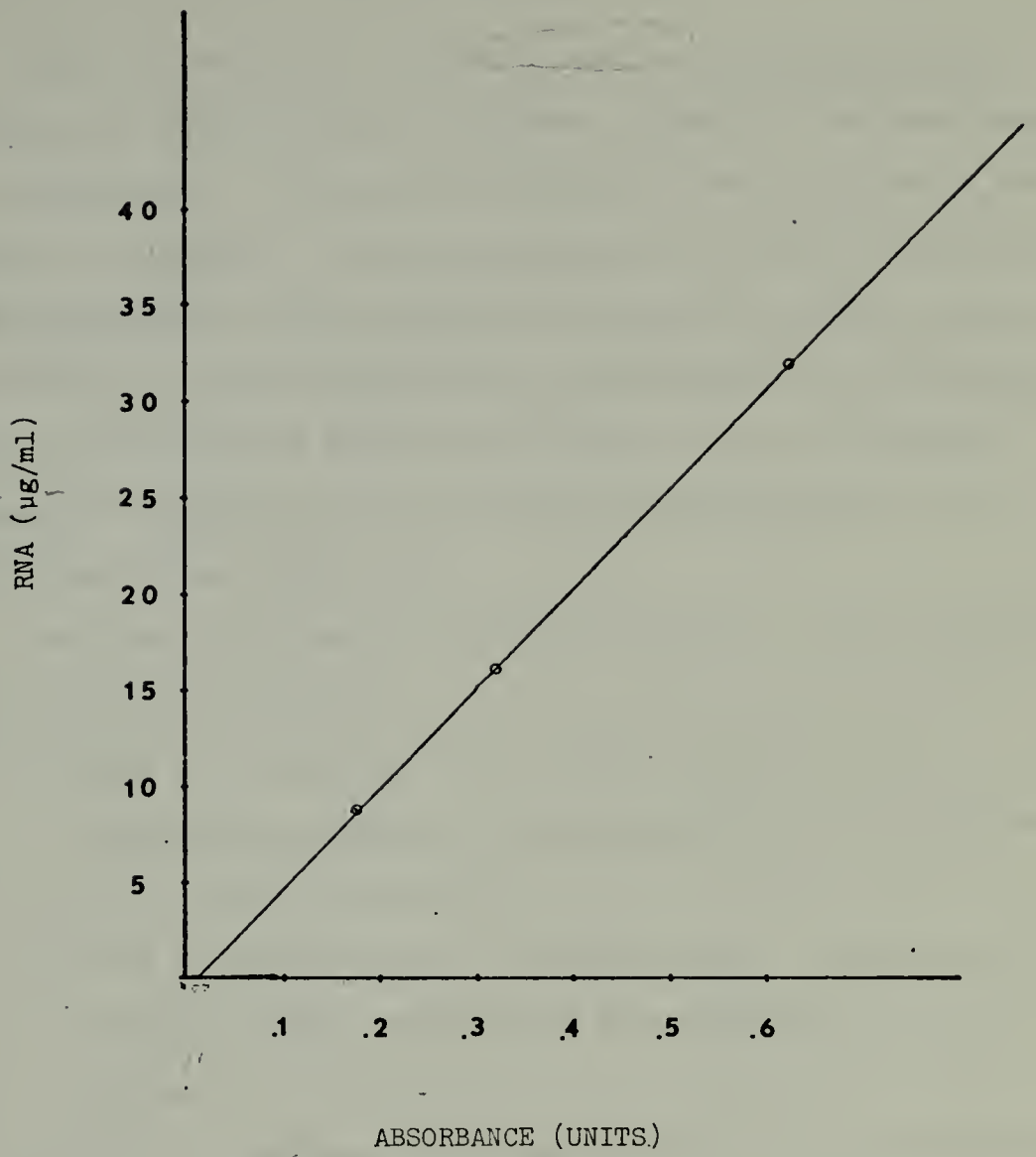


Figure 14. RNA (µ/ml) versus absorbance (units) for a yeast RNA standard.

IV. RESULTS

Data was obtained on five groups of T. californicus, each group representing a different stage in the development of a population. RNA and DNA analyses were applied to each group in duplicate. Each experimental group was composed of approximately 500 individuals (about 200 mg dry weight body mass). Growth stages were established by the relative size of individuals within each group, or by collecting copepods from splash pools predominated by young or old individuals (see Table I).

The five experimental groups consisted of the following collections of copepods:

(1) The old group was taken from a natural wild population observed to have existed over a period of at least two months.

(2) The young group was collected from a new splash pool and the older individuals were removed from the group.

(3) The all-gravid-female group was made by collecting gravid females from several splash pools.

(4) An all-but-gravid-female group was then composed of the individuals left after the gravid females were removed from those collections.

(5) A group of undetermined mixture was taken from a wild population found in the splash zone.

Table I. A description of the composition of the five experimental groups used in the analysis.

GROUP	DESCRIPTION
old	100 gravid females per 1000--mostly large in size (2-3 mm.)
young	10 gravid females per 1000--many small individuals (1 mm. or less) many mating pairs
all gravid females	all late stage gravid females (black egg sacks)
all but gravid females	mixed population from which gravid females were removed
mixed	natural population in late stage of development but not fully documented

The results of the duplicate experimental runs are shown in Table II. The all-gravid female group had the highest RNA/DNA ratio of 4.62. This was followed by the "young" group with a ratio of 3.84. The "mixed" group had a ratio of 3.15 and was followed by the all-but-gravid-female group with a ratio of 2.16. The "old" group had the lowest ratio of 1.082.

Run 2 was taken from the same homogenate as the first run, but was performed after the completion of the first run. Table III indicates the precision of the measurements made.

Possible protein carryover in the RNA extraction was estimated by taking UV absorbance readings at 260 nm and 280 nm for the range of protein standards. The results are given in Table IV. Munro and Fleck [1969] ran absorption spectra for pure RNA, a RNA fraction contaminated with peptide but digested as in their experimental procedure, and the peptide material after digestion. The results are shown in Figure 15 and will be used in conjunction with the data in Table IV to establish the status of protein carryover in the RNA extraction procedure.

A plot of RNA to DNA ratios versus population age is shown in Figure 16.

Table II. DNA and RNA values in micrograms per milliliter for all the experimental data taken.

GROUP	DNA RATIO RNA	
	RUN I	RUN II
old	$\frac{6.82}{6.29} = 1.084$	$\frac{6.80}{6.30} = 1.079$
young	$\frac{43.28}{11.27} = 3.840$	$\frac{43.24}{11.15} = 3.843$
all gravid females	$\frac{19.414}{4.20} = 4.622$	$\frac{9.707}{2.098} = 4.627$
all but gravid females	$\frac{11.72}{5.43} = 2.158$	$\frac{5.095}{2.361} = 2.158$
mixed	$\frac{25.88}{8.20} = 3.156$	$\frac{19.56}{6.20} = 3.155$

*Run II was performed separately from Run I on a portion of the original homoginate.

Table III. DNA and RNA ratios in micrograms per milliliter to micrograms per milliliter for both experimental runs, and the value of their mean and mean deviation.

GROUP	RUN I	RUN II	MEAN	MEAN DEVIATION
old	1.084	1.079	1.0815	± .0025
young	3.840	3.843	3.8415	± .0015
all gravid females	4.622	4.627	4.6245	± .002
all but gravid females	2.158	2.158	2.158	± .0001
mixed	3.156	3.155	3.155	± .0004

Table IV. Absorption values for a differential UV absorption analysis of a bovine serum albumin protein standard taken at 260 nm and 280 nm.

PROTEIN CONCENTRATION	ABSORPTION	
(μg/ml)	260 nm.	280 nm.
250	.409	.428
125	.211	.231
62.5	.102	.110
32.25	*----	.03

*below minimum sensitivity level

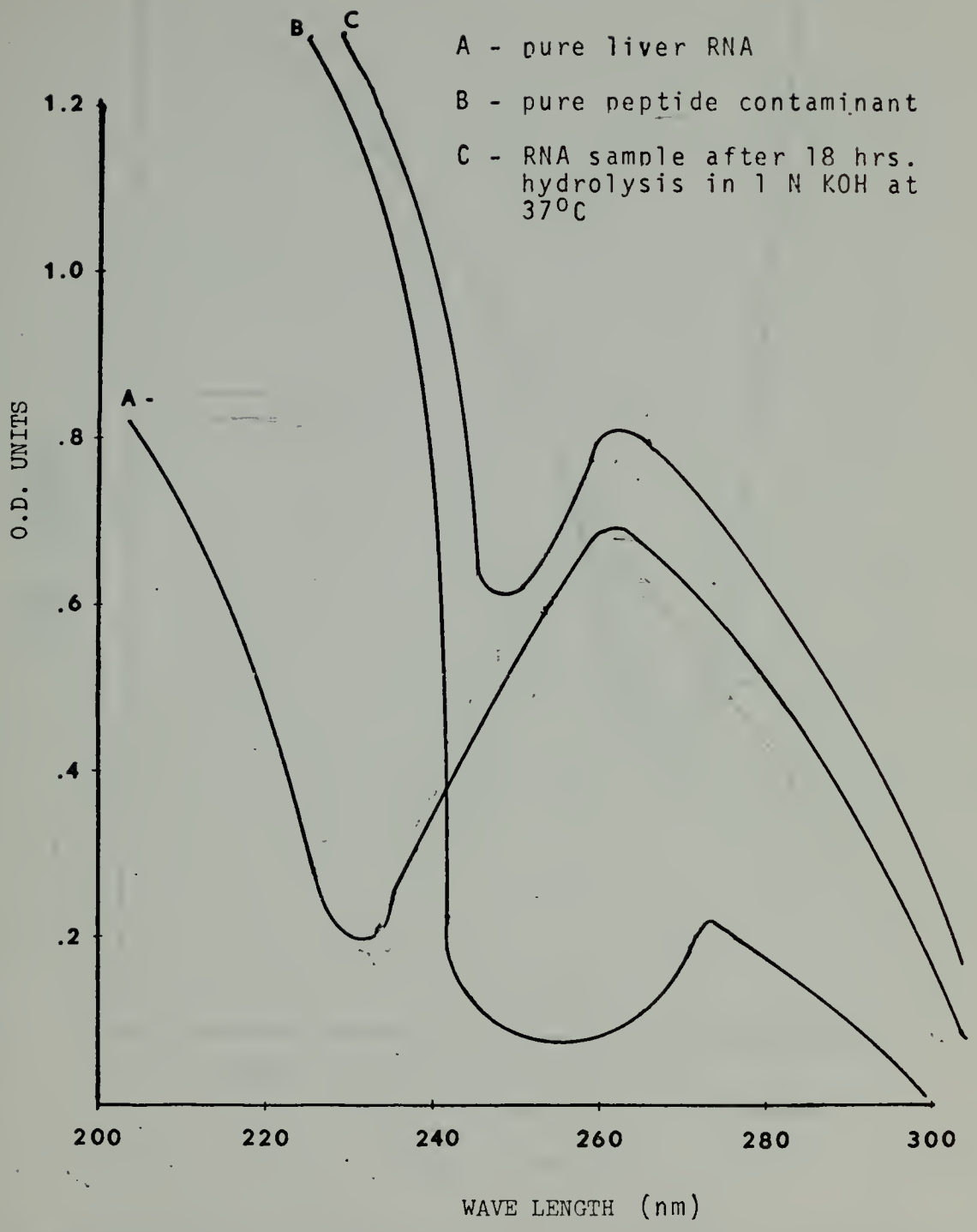


Figure 15. An absorption spectrum of pure liver RNA, pure peptide contaminant, and a RNA sample after 18 hrs. hydrolysis in 1 N KOH at 37°C, to demonstrate the effect that protein carried over with the RNA extract from the PCA-precipitated homogenate has on RNA absorption readings taken at 260 mn (after Munro, 1969).

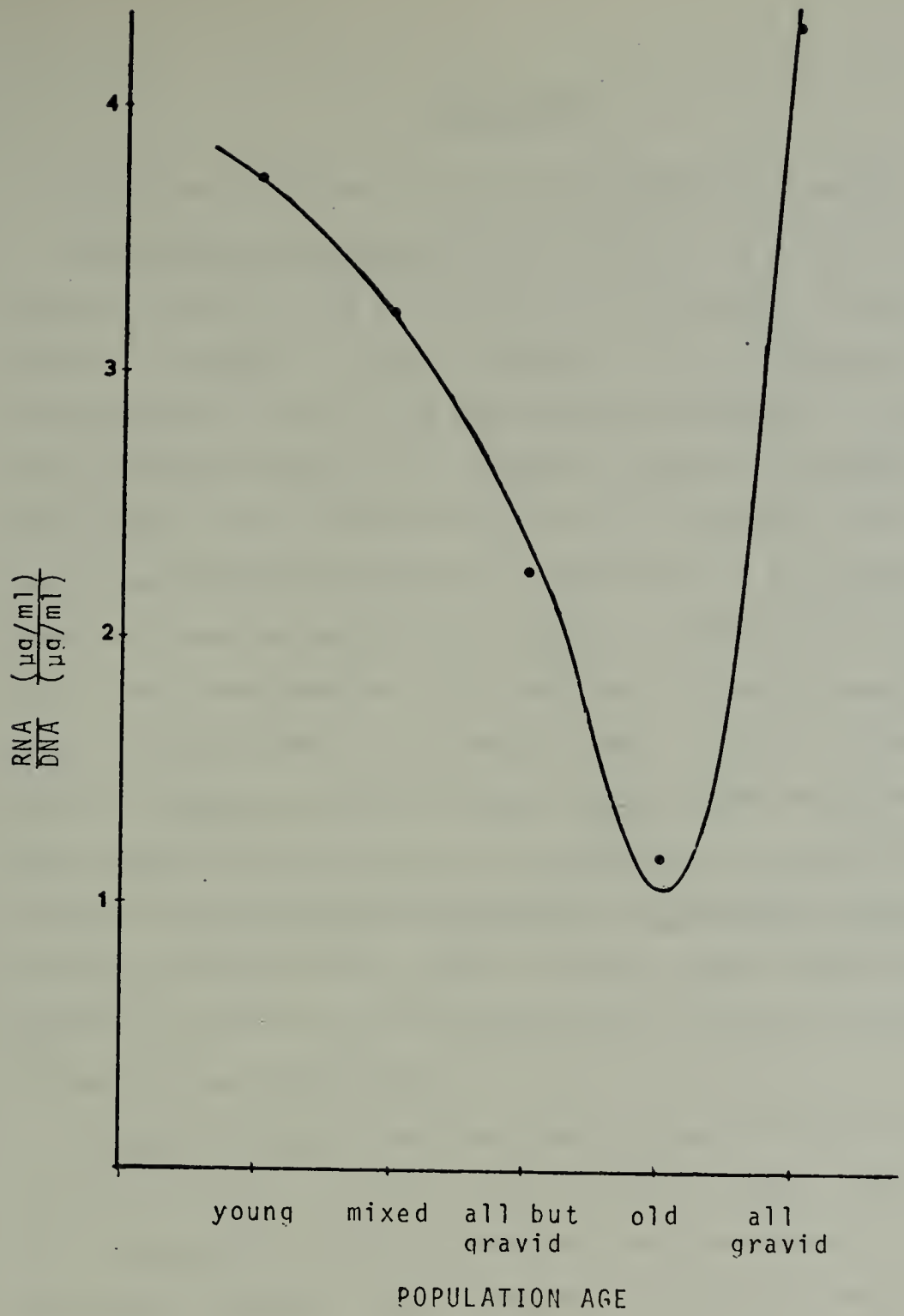


Figure 16. RNA to DNA ratios in micrograms per milliliter to micrograms per milliliter versus population age of natural occuring populations of T. californicus.

V. DISCUSSION

A. TIGRIOPUS CALIFORNICUS AS AN EXPERIMENTAL ORGANISM

Tigriopus californicus is a harpacticoid, supra-littoral benthic, copepod that is related to the pelagic, planktonic, calanoid copepods. It was important to use an organism that was convenient, but at the same time was similar to typical zooplanktonic species. The Copepoda comprise over 60% of the pelagic animal families and are, as such, the most common zooplankton in number. Populations of T. californicus were very easy to obtain since they can only live in splash pools, and their species occur exclusive of any other species along the west coast of California [Egloff, 1966]. Other kinds of organisms generally lack these characteristics, so that field collections made on splash pools contained for the most part Tigriopus californicus. Tigriopus californicus is alos a hardy species. They tolerate large temperature, salinity, and food ranges making them an excellent specimen to keep in the laboratory.

T. californicus hatch from eggs and grow through six navpliar and six copepodid stages [Egloff, 1966]. Each stage can be characterized by size and by the development of distinctive features such as setae. Thus growth stages can be readily estimated by microscopic examination of the individuals present in the population. Also the location of the splash pools can give some insight as to the age of the

population. Those pools well above the mean high water mark contained more older and more mature individuals than fresh pools. These pools remain undisturbed the longest due to their position, and allow the copepod population to develop since the copepods are not continually being washed away. The extent of the algae development in a pool can be related to the copepod population age. These characteristics of T. californicus were of additional value in the use of T. californicus as experimental organisms.

B. CHEMICAL PROCEDURE

The chemistry as modified by Munro is presumed to be the best procedures that are now available. The mean deviation for these procedures (Table III), was ± 0.002 ratio units. The high precision of the methods is apparent in the standard curves (see figures). Both DNA and RNA standards produced a negligible amount of scatter in three separate standard curve determinations (see Figures 13 and 14). At the end of the five-month experimentation period standards were run against the initial stock solutions of RNA, DNA, and protein. The absorbance was found to be the same as that originally recorded.

Protein carried over into the RNA extract from the RNA digestion step was of concern to Sutcliffe in his work [Sutcliffe, 1965]. He pointed out this fault in Dagg and Littlepage's work in personal correspondence to Dr. Eugene Traganza, and recommended that a Lowry protein determination

be performed on the RNA extract in order to correct the value of RNA absorption for absorption from protein carryover. In modifying the basic Schmidt-Thannhauser procedure Munro went to great lengths to insure that the concentration of base, and the time and temperature of digestion would be sufficient to hydrolize all the RNA, but not the tissue proteins present. As a result one can expect not to have protein interference in the RNA absorption. However, to insure that negligible protein carryover was occurring, the UV absorption of bovine serum protein standards was made at 260 nm and 280 nm (Table IV) to compare with Munro's results (Figure 15) and the results of the RNA extraction absorption readings. The low level of absorptions obtained at 280 nm from relatively high concentrations of pure protein indicates that the higher absorptions read at 280 nm are due to the "tail" of the RNA 260 nm spectrum curve, not to protein interference. Figure 11 from Munro indicates this RNA "tail effect". Thus by ensuring that all the readings at 280 nm were significantly less than the readings at 260 nm and that these 280 nm readings follow the trend in RNA readings at 260 nm, protein interference is not significant to the RNA absorptions. Munro's standard curves shown in Figure 15 indicate that the RNA hydrolysis step, if performed for 15 min. in 0.3 N KOH at 37°C, will not appreciably hydrolyze any of the phospholipids and tissue proteins present in the extract.

There is a degradation factor involved with the analysis of DNA. Nuclear DNA will decompose at normal room temperatures.

Early DNA measurements indicated this when care was not taken to minimize the time between the filtering of the copepods and the time when homogenization took place. Accordingly the analysis should proceed as rapidly as possible and the homogenation must occur at low temperatures.

C. PREVIOUS WORK

Sutcliffe [1969] showed a positive trend between RNA concentration and growth rate during some organisms most rapid growth stage. Dagg and Littlepage [1972] pointed out that this trend generally holds, but only during the most rapid period of growth. They maintained that the trend was non-specific at other growth times. Sutcliffe [1969] himself has admitted that his data lacked a sufficient number of points to make the RNA-growth rate trend specific to an entire life-cycle as well as to life cycles of different organisms. Thus the RNA-growth rate relationship can only be considered well defined during the organisms most rapid growth period. Much of the work done along this line has involved the use of synchronous, laboratory cultures. Apparently no one has tried to extend the RNA-growth rate trend to a natural occurring population of mixed organisms or even of mixed growth stages, i.e., some young, some old, some maturing. From these facts the author feels that if a meaningful and workable relationship between RNA and growth rate is to be obtained naturally occurring mixed populations should be used in the analysis.

Dagg and Littlepage did not check their work for protein carryover in the RNA extraction step. Though it was expected that not much error would be introduced in the RNA absorption, it was necessary to ensure that the chemical technique was not producing an erroneous RNA concentration. In personal correspondence to Dr. Eugene Traganza, Sutcliffe stated that he found up to 50% error in Dagg's first RNA readout method due to protein carryover. This source of error according to Sutcliffe could easily account for the data scatter that Dagg and Littlepage mentioned in their work. Again it becomes obvious that protein interference must be accounted for or must be prevented.

D. RNA/DNA VERSUS GROWTH STAGE

Figure 16 presents the relationship between RNA/DNA and growth stage as developed in this work. The arrangement of the five points is not fixed to the sequence shown in Figure 16, but can arbitrarily be shifted to any position. The arrangement shown was used since it is consistent with the RNA-growth rate relationship.

The "young" group has the highest ratio of any of the mixed groups which indicates that the RNA concentration on a cellular level is the highest of any of the mixed groups. This result is expected in that the individuals in this category are young and immature. They are going through a definite growth sequence which implies that the RNA concentration should be going through a definite high level.

The "young" group was not a natural population. Young individuals were added to over-emphasize the "youngness". The intentions here were to bracket the other mixed populations of various ages. The "old" group was taken from a wild population and was experimentally tested as a naturally occurring group. It had the lowest RNA-DNA ratio of any of the groups. Again the result would be expected from knowledge of the RNA-protein synthesis relationship. The "old" group was mostly mature individuals with a few late stage gravid females. It appeared that most of the females had dropped their egg sacs since there were a large number of large females present without egg sacs. The "mixed" group fits in between the old and young groups. Again this trend follows since the "mixed" group could not be as young as the "young" group, but yet was not as "old" a population as was the "old" group. The lack of documentation for this group was unfortunate, for a good description would tell how close to either extreme, young or old, the group should fit. What little was known about this group indicated that it was closer to a young population than an older one and this evidence is supported by its RNA-DNA ratio. The all-but-gravid-female group was the remaining individuals from a population originally dense in gravid females. Because the population was very dense in gravid females it was presumably in a later stage of development than the young group which would explain its lower RNA/DNA ratio. In fact, this group could be likened to the old group that had few gravid females.

The position of this group before the gravid females were removed is indeterminate, but could be estimated to fall between the "young" and "old" group boundaries. The all-gravid-female-group represents the highest RNA/DNA ratio. It is not known whether the high RNA concentration is due to the eggs or to the females carrying the eggs. The work of Vickers and Mitlin [1965-66] showed that boll weevil eggs contained very little RNA so that it might be surmised that the high RNA concentration is related to the gravid females' high protein production rate. Since eggs are basically protein, this idea seems to be reasonable. The positioning of the point representing the all-gravid-female-group is quite debatable. The all-gravid-female-group can be looked at as representing the maximum population potential. This view point would require that the all-gravid-female-group be placed after the "old" group on the population age axis since a population must mature before it can reproduce. The all-gravid-female-group can also be considered to represent the group with the greatest protein synthesis rate. This viewpoint would require the placement of the group before the "young" group on the population age axis. The all-gravid-female-group is not a real population and its placement is strictly arbitrary. What is important is that a definite trend of ratio versus growth stage has been established. Younger populations have a higher RNA/DNA ratio than do older populations.

Another approach to explaining the shape of Figure 16 can be made by comparing population dynamics to the characteristics of an individual's growth. Dagg and Littlepage's Artemia salina culture A exhibited a Sigmoid growth curve (see Figure 17). A rough plot of their RNA/DNA ratios for the same culture is shown in Figure 17. Superimposing the two curves on similar axes (see Figure 17) shows the relationship of their RNA/DNA ratios to the Sigmoid growth stage that their synchronous culture was in. Their trend was for a decreasing RNA-DNA ratio in stage I with a minimum somewhere in stage II. From that point on through stage III the trend is for an increasing RNA-DNA ratio. When the author's five different growth stage points are placed on the same growth curves in accordance with the reasoning explained earlier in this section, a very similar curve emerges (see Figure 17). This results in the implication that individual growth tendencies can be related to population growth tendencies. There is still a large, important area that is in question, and that occurs at the end of Sigmoid stage III. The trends of the natural populations were not measured in this stage, and the synchronous cultures usually die off after they reach maturity. Data obtained in this phase would greatly help to clarify the actual shape of the growth stage to RNA/DNA curve. Too little was known about the dynamics of Tigriopus populations to predict a trend for this stage, nor was the time available to pursue measurements in this area.

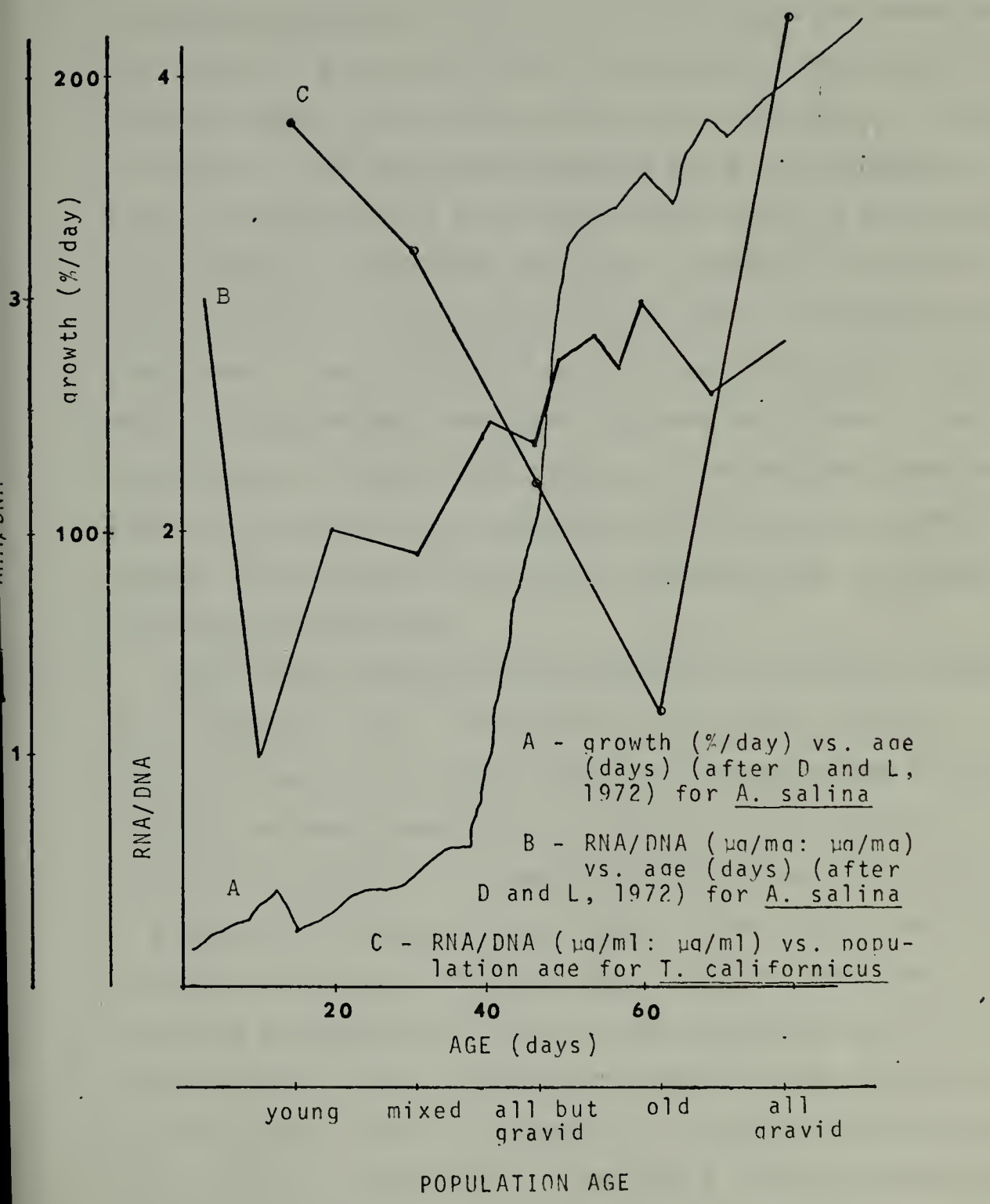


Figure 17. A superposition of Dagg and Littlepage's (1972) A. salina plots of growth (%/day) versus age (days) RNA/DNA (µg/mg: µg/mg) versus age (days), and the author's T. californicus RNA/DNA (µg/ml: µg/ml) versus population age.

E. APPLICABILITY

Due to the large amount of time involved in collecting and preparing groups for analysis and the time necessary for the analysis (3 hours per trial), data had to be limited to boundary values. More data points would have given a clearer picture as to how the RNA/DNA-growth stage trend changes. What is important from this work is that there is a definite trend existing between RNA and growth stages of a natural, mixed population of Tigriopus. It is felt that further work along these lines of RNA/DNA-growth stage indications would better elucidate the trend that has been established. The actual value of this trend, though, is not without question. Even if the trend could be established through all growth stages, it would still have to be re-established for actual zooplankton populations.

Total RNA concentrations may not be as sensitive a measure as is needed to get a firm grasp on zooplankton growth. Eighty percent of the RNA analyzed was ribosomal RNA which is a fairly constant quantity over the life span of any one species [Fan, 1961]. The quantity of ribosomal RNA present in a cell is a function of cell size. Thus mature cells have a constant amount of ribosomal RNA. tRNA and mRNA are generated as needed in a cell so that at any one time ribosomal RNA is the largest contributor to the total amount of RNA present. This is a result of the molecular structure of the various forms of RNA. tRNA has a molecular weight of 10,000 to 30,000; mRNA has a molecular weight of 40,000 to

60,000; and ribosomal RNA ranges up to 1,300,000 [Fan,1961]. Thus ribosomal RNA is the major contributor to RNA quantitative analysis and could act to mask out changes in the amounts of tRNA and mRNA over a cell's life.

F. FUTURE METHODS

The analysis could have been made more sensitive by measuring chemical, cellular quantities that are more vital to the actual rate of protein synthesis. Pease [1973] suggested that ribonuclease be used. Messenger RNA may be more directly indicative of protein synthesis than is total RNA. An enzyme that triggers protein synthesis, e.g., RNA synthetase or one that is directly involved in constructing all proteins may be a more specific growth indication than total RNA. The problem remains to isolate this indicator and to devise a chemical analysis that will accurately and in real time give a measure of the indicators concentration. Most important is the knowledge that a chemical measurement of some cellular indicator common to all zooplankton, has the potential to provide an accurate, reliable measurement of zooplankton growth rates, biomass or population. More research is necessary to determine the best course to pursue.

Once this is done it could become possible to adapt the chemical analysis to an automated method. This adaption would lead to real-time, in situ growth rate measurements that could lead to the continuous, accurate prediction of zooplankton populations in the sea and ultimately the prediction of sound scattering through the food chains.

G. A CRITIQUE

The author's own work indicates the importance of employing control groups in studying population characteristics, for his lack of using such leaves his results in some question as to interpretation. Employing controls would lead to more specific results. A control population developed in the laboratory and sampled at periodic intervals would give the RNA/DNA ratios more identification, and would provide information to better determine the growth stage of a natural population. All phases of collection and analysis should be well documented to provide statistics that are complete and relevant. The author could have made his statistics more complete and meaningful by fully documenting all the stages of this work, and especially by paying particular attention to the collection stage. By carefully noting the physical location, the algae development, and the general size and number of copepods in the splash pools, a good idea of the population age can be formulated.

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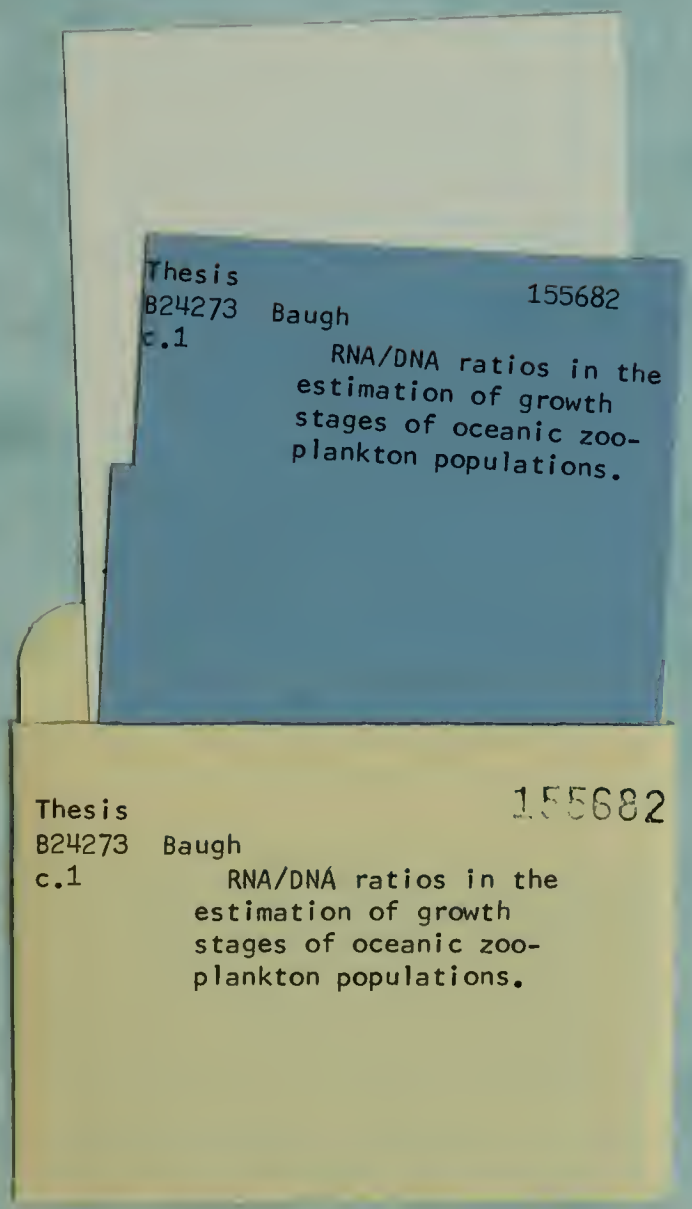

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